



## Heme transport exhibits polarity in Caco-2 cells: evidence for an active and membrane protein-mediated process

Aliye Uc,<sup>1</sup> John B. Stokes,<sup>2</sup> and Bradley E. Britigan<sup>2</sup>

Departments of <sup>1</sup>Pediatrics and <sup>2</sup>Internal Medicine, Veterans Administration Medical Center, The University of Iowa, and the Roy J. and Lucille A. Carver College of Medicine, Iowa City, Iowa 52242

Submitted 8 April 2004; accepted in final form 6 August 2004

Uc, Aliye, John B. Stokes, and Bradley E. Britigan. Heme transport exhibits polarity in Caco-2 cells: evidence for an active and membrane protein-mediated process. *Am J Physiol Gastrointest Liver Physiol* 287: G1150–G1157, 2005. First published August 12, 2004; doi:10.1152/ajpgi.00157.2004.—Heme prosthetic groups are vital for all living organisms, but they can also promote cellular injury by generating reactive oxygen species. Therefore, intestinal heme absorption and distribution should be carefully regulated. Although a human intestine brush-border heme receptor/transporter has been suggested, the mechanism by which heme crosses the apical membrane is unknown. After it enters the cell, heme is degraded by heme oxygenase-1 (HO-1), and iron is released. We hypothesized that heme transport is actively regulated in Caco-2 cells. Cells exposed to hemin from the basolateral side demonstrated a higher HO-1 induction than cells exposed to hemin from the apical surface. Hemin secretion was more rapid than absorption, and net secretion occurred against a concentration gradient. Treatment of the apical membrane with trypsin increased hemin absorption by threefold, but basolateral treatment with trypsin had no effect on hemin secretion. Neither apical nor basolateral trypsin changed the paracellular pathway. We conclude that heme is acquired and transported in both absorptive and secretory directions in polarized Caco-2 cells. Secretion is via an active metabolic/transport process. Trypsin applied to the apical surface increased hemin absorption, suggesting that protease activity can uncover a process for heme uptake that is otherwise quiescent. These processes may be involved in preventing iron overload in humans.

heme oxygenase; alveolar epithelial cells; small intestine; enterocyte; tin protoporphyrin

IRON PLAYS A CRITICAL ROLE in the normal physiology of living organisms, but it is also a source of cytotoxic free radicals. Because humans are unable to excrete excess iron, the body's iron balance is regulated through controlling its intestinal absorption (10). The majority of the body's iron is in the form of heme, which participates in the structures of many important enzymes, but can also be a source of reactive oxygen species (3, 21).

In North America and Europe, two-thirds of the dietary iron is present as ferric iron and one-third as heme (7). The majority of dietary ferric iron is not bioavailable. Therefore, the two-thirds of iron absorbed through intestines is in the heme form. Because of its prooxidant effects and its role in providing the majority of the body's iron, heme absorption and distribution should be carefully regulated (7, 24). However, little is known about the mechanism of heme absorption through the intestinal mucosa (35, 47).

Heme enters the enterocyte as an intact metalloporphyrin and the uptake is not competitive with nonheme iron, indicating that acquisition occurs via a different mechanism (11, 35, 47). It has been suggested that heme enters endothelial cells, murine erythroleukemia cells, embryonic cells, and HepG2 hepatoma cells via a heme-receptor-mediated internalization process (3, 13, 14, 16, 17, 33). It is not known whether heme enters intestinal epithelial cells via a similar mechanism, but a poorly characterized brush-border heme receptor has been suggested to be present in pig and human intestine (18, 19). Others suggested that the heme acquisition process is independent of a receptor, and heme traverses the membranes via passive pathways (31).

Once inside the cell, heme is degraded by an inducible enzyme, heme oxygenase-1 (HO-1), with the release of inorganic iron (37). Inorganic iron formed by heme degradation is believed to compete with nonheme iron acquired by the cell through the diet or body stores. Although human studies have been useful in elucidating the effects of dietary components on heme absorption (22–25), they are not suitable to study cellular and biochemical pathways involved in heme absorption. Most of the information about heme acquisition comes from studies on bacteria that use heme as a source of iron (12).

Caco-2 cells are an established model of intestinal epithelial cells (20). They form a polarized monolayer and, after confluency, differentiate to develop many characteristics of the small bowel epithelium. With their ability to absorb iron (2) and synthesize HO-1 (6, 44), Caco-2 cells could be a good model for assessing heme trafficking. Recent studies suggested the presence of a heme receptor in Caco-2 cells (46), which may be regulated by the cellular iron status (15). However, the actual heme transport mechanisms have not been identified.

Our study shows that heme is capable of being acquired and transported in both the absorptive (apical to basolateral) and secretory (basolateral to apical) directions in Caco-2 cells via an active metabolic/transport process. Trypsin increases heme absorption, suggesting the presence of a protein that negatively regulates heme uptake by the apical membrane. These events may be important mechanisms to prevent iron overload in humans.

### MATERIALS AND METHODS

**Tissue culture.** Caco-2 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and were used between passages 30 and 65. Stock cultures were grown to confluency at 37°C in 5% CO<sub>2</sub> using DMEM (Mediatech, Cellgro, Herndon, VA) con-

Address for reprint requests and other correspondence: A. Uc, 2865 JPP Pediatrics, Univ. of Iowa Health Care, 200 Hawkins Drive, Iowa City, IA 52242 (E-mail: aliye-uc@uiowa.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

taining 4.5 g/l glucose, 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 15 mM HEPES. Fresh medium was added every 2 days. Cells were released from stock plates by 3–5 min of 0.25% trypsin-0.1% EDTA treatment and plated at 30,000 cells/cm<sup>2</sup> on polyester filters (Transwells; Costar, Cambridge, MA). Development of a confluent monolayer was confirmed when transepithelial resistance (TER) was stable for two successive days (~13–16 days after confluency).

Calu-3 cells, a line of human airway epithelial cells derived from lung adenocarcinoma were kindly provided by Dr. Michael Welsh's core facility (University of Iowa, Iowa City, IA) after they were grown on 12-mm Millicell PCF filters (Millipore, Bedford, MA) to confluency and developed tight junctions in an air-liquid interface.

H441 cells, an alveolar type II epithelial cell line, were grown in RPMI 1640 with 6% FCS, 5 µg/ml insulin, 5 µg/ml transferrin, 10 nM sodium selenite, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded at 30 µg DNA/cm<sup>2</sup> on 12-mm Millicell PCF filters that had been pretreated with human placental collagen. The study was done after H441 cells became confluent as described (40).

T84 cells were obtained from ATCC and grown to confluency at 37°C in 5% CO<sub>2</sub> using 1:1 mixture of DMEM and Ham's F-12 medium containing 1.2 g/l sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES, and 0.5 mM sodium pyruvate. Fresh medium was added every 2 days. Cells were released from stock plates by brief trypsin-EDTA treatment and plated at 30,000 cells/cm<sup>2</sup> on 12-mm Millicell PCF filters. The experiments were conducted after the cells became confluent and developed a stable TER (~18–21 days after seeding).

**Preparation of porphyrins.** Hemin (3 mM, Sigma-Aldrich, St Louis, MO) was prepared in 1 N NaOH and diluted to 0.5 mM in phenol red-free MEM with L-glutamine (GIBCO Invitrogen, Carlsbad, CA) and 10% FCS. The pH was adjusted to 7.4 by the slow addition of 12 N HCl. For convenience, we will use heme (Fe<sup>2+</sup>) and hemin (Fe<sup>3+</sup>) interchangeably. Tin protoporphyrin (SnPP) was obtained from Frontier Scientific (Logan, UT) and diluted in 0.1 N NaOH.

**Cell viability assay.** The methyl thiazol tetrazolium (MTT) assay was used to determine cell viability. This assay relies on production of formazan by the action of mitochondrial enzymes on MTT by living cells, and correlates well with other measures of cell number (26). Cells were exposed to the desired concentration of hemin for 12–24 h, and then incubated with 0.5 mg/ml MTT (Sigma-Aldrich) for 2 h. The formazan formed was solubilized in 2-propanol and quantitated by measuring the absorbance at 550 nm.

**HO-1 immunoblot analysis.** Proteins were separated by SDS-PAGE using 12% acrylamide resolving gels. After electrophoretic transfer to a nitrocellulose membrane, the blots were blocked in TTBS buffer (in mM: 10 Tris, pH 7.4, 130 NaCl, and 0.8 disodium EDTA plus 1% Tween 20) with 5% nonfat dairy milk for at least 1 h, and subsequently incubated for 1 h with polyclonal HO-1 antibody to human (1:2,000) (StressGen Biotechnologies, San Diego, CA) diluted in TTBS buffer. The specific protein was detected by using goat-anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (1:10,000) (Upstate Biotechnology, Lake Placid, NY). Blots were washed several times with TTBS buffer. Antibody-labeled bands were visualized by incubating the blots for 1 min with enhanced chemiluminescence (ECL substrate; Amersham, Arlington Heights, IL), and exposing Kodak XAR film for 1–5 min. The percent increase in protein induction was obtained by densitometric scanning (Alpha-Innotech, San Leandro, CA). Antibodies against catalase, CuZnSOD, and MnSOD were kindly provided by Dr. Larry Oberley at the University of Iowa. Inducible nitric oxide synthase (iNOS) antibody was purchased from BD Transduction Laboratories (San Jose, CA).

**Measurement of porphyrin transport.** After treatment of Caco-2 cells monolayers with hemin (10–250 µM) or SnPP (5–50 µM), apically or basolaterally, medium on the opposite side was collected, and transport was determined spectrophotometrically in a diod-array

UV/visible spectrophotometry (Agilent Tech, Palo Alto, CA). Hemin concentration was measured at 407 nm using hemin standards and an extinction coefficient of  $3.6 \times 10^4$  M/cm. SnPP transport was determined at 409 nm using SnPP standards and an extinction coefficient of  $12.4 \times 10^4$  M/cm. Transport was expressed as nanomoles per surface area over time. Transport experiments were carried out in phenol red-free medium, because phenol red, SnPP, and hemin absorbed UV light at similar wavelengths.

**Measurement of cell-associated hemin.** Hemin content of Caco-2 cells was determined by using the protocol described by Balla et al. (3). Confluent Caco-2 cells were treated with hemin (10–250 µM), apically or basolaterally. At the end of treatment, medium containing hemin was aspirated, and cells were washed with HBSS three times. Cells were removed with  $2 \times 0.5$  ml of formic acid washes, and heme content of formic acid solubilized cells was determined spectrophotometrically at 398 nm with an extinction coefficient of  $1 \times 10^5$  M/cm. Cell-associated hemin was expressed as nanomoles per filter over time.

**[<sup>3</sup>H]mannitol flux.** To measure paracellular permeability, 0.2 µCi/ml of [<sup>3</sup>H]mannitol (Perkin Elmer Life Sciences, Boston, MA) was applied to cell monolayers apically or basolaterally, at the same side of hemin treatment. At the end of treatment, 100 µl of medium was aspirated from the apical and basolateral sides and radioactivity was counted in a scintillation counter. [<sup>3</sup>H]mannitol flux to the opposite side was calculated as the percentage of the total isotope administered per square centimeter of surface area (38).

**Measurement of TER.** TER was measured with an ohmmeter (World Precision Instruments, Sarasota, FA) using the method described by Hidalgo et al. (28) and defined as resistance multiplied by the surface area of the membrane. The resistance of the supporting membrane and solutions was subtracted from all readings.

**Statistics.** One-way ANOVA and Tukey's method are used for comparison of different treatment groups. Results are expressed as means  $\pm$  SE. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

**Heme oxygenase induction exhibits polarity in Caco-2 cells.** We and others (6, 44) have shown that Caco-2 cells increase their HO-1 mRNA, protein levels, and enzyme activity in response to hemin in a dose- and time-dependent manner. Because intestinal epithelial cells are primarily exposed to hemin in vivo coming from the intestinal lumen, we hypothesized that the HO-1 induction would primarily occur after an apical hemin exposure and investigated Caco-2 cell responses with apical and/or basolateral hemin treatments. Confluent Caco-2 cells were treated with various concentrations of hemin (50–250 µM) and HO-1 immunoblots were obtained. These concentrations of hemin were nontoxic, as demonstrated by the MTT assay (control:  $0.59 \pm 0.01$ ; hemin 250 µM apical:  $0.61 \pm 0.01$ ; hemin 250 µM basolateral:  $0.62 \pm 0.01$ ,  $n = 4$ ,  $P =$  not significant). Caco-2 cells showed a dose-dependent response to apical or basolateral hemin exposure, but contrary to our expectations, the response was far greater if they were treated from the basolateral side (Fig. 1, A and B). Apical treatment did not have any additive effect over HO-1 induction with basolateral exposure. Hemin did not induce iNOS, catalase, CuZnSOD, and MnSOD proteins, as assessed by immunoblot analysis (data not shown).

**Cell-associated hemin after apical or basolateral hemin treatment.** We postulated that the greater HO-1 induction after basolateral treatment could be related to hemin having better access to the intestinal epithelial cell cytoplasm from the basolateral side. To test this hypothesis, we measured the

HEME TRANSPORT IN CACO-2 CELLS

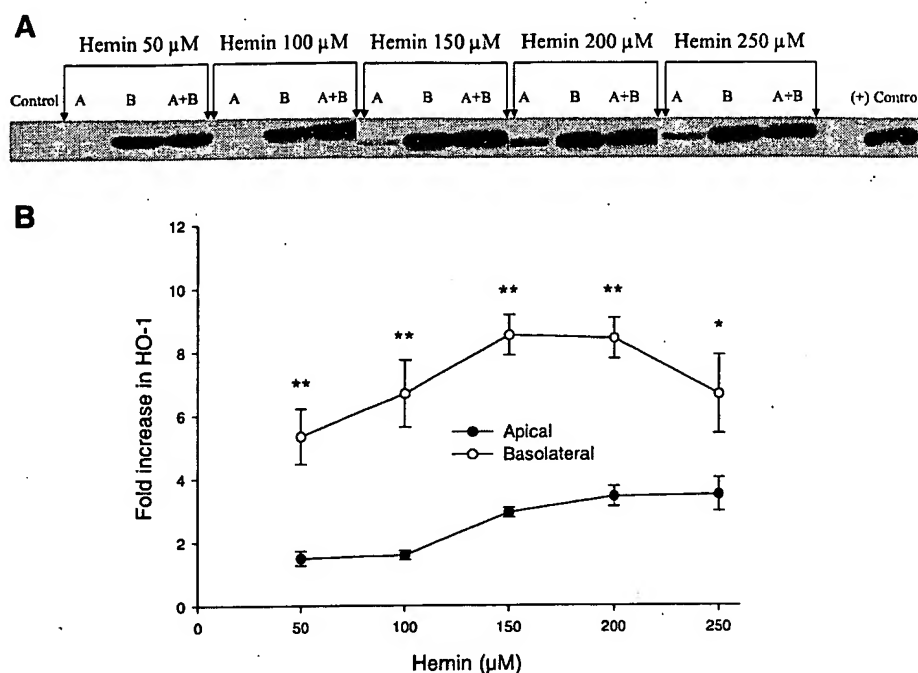


Fig. 1. Differential induction of heme oxygenase-1 (HO-1) in Caco-2 cells. Caco-2 cells grown on filters were treated with hemin from the apical side (A), the basolateral side (B), or both. Immunoblots were performed. The far right lane contains 50 ng of HO-1 protein as a positive control. Hemin-dependent induction of HO-1 in Caco-2 cells was dose-dependent when exposure was either apical or basolateral, and HO-1 induction was far greater when exposure was basolateral. B: densitometric analysis of the HO-1 immunoblots after treatment with apical or basolateral hemin for 24 h.  $n = 5$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ .

cell-associated hemin (CAH) levels in Caco-2 cells (3). Cells were grown in the same conditions described above and treated with hemin (25–250  $\mu$ M) apically or basolaterally. CAH was not different with apical compared with basolateral hemin exposure at 24 h (Fig. 2A). CAH was also examined over time with apical (100  $\mu$ M) or basolateral (100  $\mu$ M) hemin exposures (Fig. 2B). CAH levels were higher with apical hemin treatment initially (15 min, 30 min, 1 h, 2 h). These are time points when cellular HO-1 levels are still low and heme metabolism is probably also low (44). CAH levels were not different between apical and basolateral treatments after 6 h of exposure. Control cells had no endogenous heme detectable with this technique.

**Hemin transport exhibits polarity in Caco-2 cells.** Because Caco-2 cells show a greater induction of HO-1 with basolateral rather than apical hemin exposure, it is possible that transport mechanisms are different or a heme transporter is differentially expressed on the apical vs. basolateral side of the cells. To investigate the hemin transport, confluent Caco-2 cells grown on filters were treated with hemin (10–250  $\mu$ M) from the apical or basolateral side. Hemin was quantitated in the medium from the opposite side spectrophotometrically, as described in MATERIALS AND METHODS. As shown in Fig. 3A, hemin secretory transport was more rapid than absorptive transport at all hemin concentrations. Hemin absorption and secretion (100  $\mu$ M) were very low and not different the first 1 h of exposure. Hemin secretion continued with time; hemin absorption approached a plateau after 2 h (Fig. 3B). We observed that SnPP was also more rapidly secreted than absorbed, suggesting that differential secretion was a common feature of protoporphyrins, regardless of the associated metal (Fig. 4).

**Effect of hemin on paracellular permeability.** During these experiments, we noticed that TER decreased if higher concentrations of hemin ( $\geq 200$   $\mu$ M) were applied to the basolateral side (Fig. 5A). If this decrease in TER was the result of

increased permeability of the junctional complexes, a portion of the secretory transport at these hemin concentrations could be via enhanced diffusion through this pathway.

To determine the extent to which hemin secretory transport was related to increased paracellular permeability, we measured [ $^3$ H]mannitol flux across the monolayer. [ $^3$ H]mannitol was applied to the same side as hemin and its flux to the opposite side of the monolayer was determined. Cell monolayers did not demonstrate increased permeability to [ $^3$ H]mannitol if hemin was given apically (Fig. 5B). In contrast, when hemin concentration was 200  $\mu$ M or greater on the basolateral side, [ $^3$ H]mannitol flux increased. Together, the results of the mannitol flux, the TER measurements, and the unidirectional fluxes indicate that the majority of the secretory hemin flux at concentrations of  $<200$   $\mu$ M did not occur via a paracellular pathway, but rather via a transcellular pathway.

**Hemin secretion is active.** The difference between the secretory and absorptive hemin flux (Fig. 3A) suggested that secretion might be an active process. We tested this idea in two ways. First, we examined the temperature dependence of the transport processes. Confluent Caco-2 cells were treated with hemin (250  $\mu$ M apically or 100  $\mu$ M basolaterally) at 37 or 4°C for 6 h. Cell viability at these conditions was confirmed with MTT assay. Both absorptive and secretory fluxes slowed considerably at 4°C, but the secretory flux slowed to a much greater degree than the absorptive flux (Fig. 6, A and B). These results are consistent with the idea that at least the secretory flux was active. We therefore tested whether hemin could be transported against a concentration gradient. We treated confluent Caco-2 cells with the same concentrations of hemin apically and basolaterally. After 24 h, hemin concentrations on both sides were measured spectrophotometrically (Table 1). Hemin was more concentrated on the apical side, demonstrating that secretory transport occurred against a concentration gradient and was therefore active.

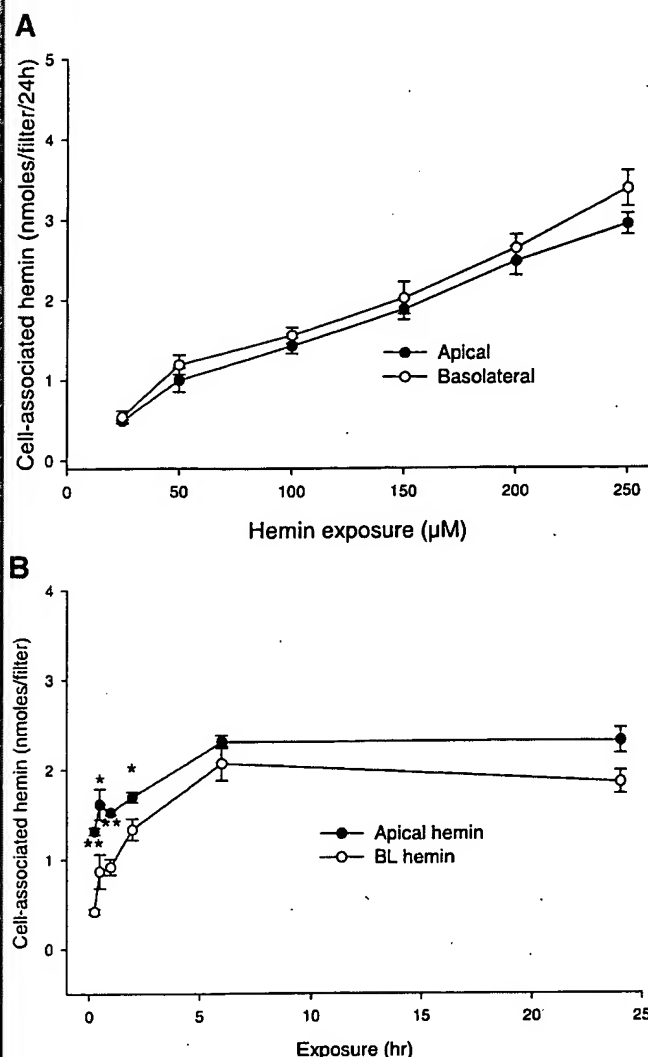


Fig. 2. Cell-associated hemin (CAH) in Caco-2 cells. Cells were removed with formic acid, and cellular heme content was measured spectrophotometrically. A: dose response: Caco-2 cells were treated with 25–250  $\mu$ M hemin from the apical or basolateral side for 24 h. CAH was not different with apical or basolateral exposure ( $n = 10$ ,  $P =$  not significant). B: time course: CAH after apical (100  $\mu$ M) or basolateral hemin (100  $\mu$ M) was examined at 15 min, 30 min, 1 h, 2 h, 6 h and 24 h. CAH was higher with apical hemin exposure  $< 6$  h.  $n = 4$ ,  $*P < 0.05$ ;  $**P < 0.01$ .

**Porphyrim binding to proteins.** Because heme is bound to proteins in biological systems (8, 34) and our experiments are conducted in serum-containing medium, we determined the amount of free hemin in our experimental conditions. Various concentrations of hemin were prepared in medium (phenol red-free MEM) containing FCS (5 or 10%) or BSA (1, 2, or 4 mg/ml). Hemin preparations were centrifuged in Centricon tubes (Millipore, Bedford, MA) (molecular weight cut-off: 10,000) at 3,000  $g$  (Beckman centrifuge) for 60 min to separate serum-bound hemin from its free form. The spectrophotometric analysis of the ultrafiltrate showed no absorbance for hemin at 400 nm, suggesting that in our experimental conditions, hemin was essentially 100% bound to serum proteins or albumin. If cells were treated with hemin in the absence of FCS, a precipitation was noticed in the chambers.

We determined that in our experimental conditions (10% FCS), SnPP was 74% protein-bound. Binding of SnPP to albumin increased in a dose-dependent manner (61% at 1 mg/ml BSA, 64% at 2 mg/ml BSA, 76% at 4 mg/ml BSA).

**Heme absorption is activated by trypsin.** To determine whether heme transport could be modified by altering an apical membrane protein(s), monolayers were incubated with trypsin (0.0025%)-EDTA (0.001%) for 5 min apically or basolaterally before measuring hemin flux. The monolayers were then washed with HBSS three times, and the solution was then changed to phenol red-free medium containing hemin (250  $\mu$ M apically or 100  $\mu$ M basolaterally) for 30 min to 6 h. Trypsin increased apical hemin absorption by threefold, whereas denatured trypsin had no effect (Fig. 7A). Trypsin applied to the basolateral solution had no effect on hemin secretion (Fig. 7B). Trypsin did not change TER or [ $^3$ H]mannitol flux across the monolayer (Table 2). Preincubating cells with apical trypsin

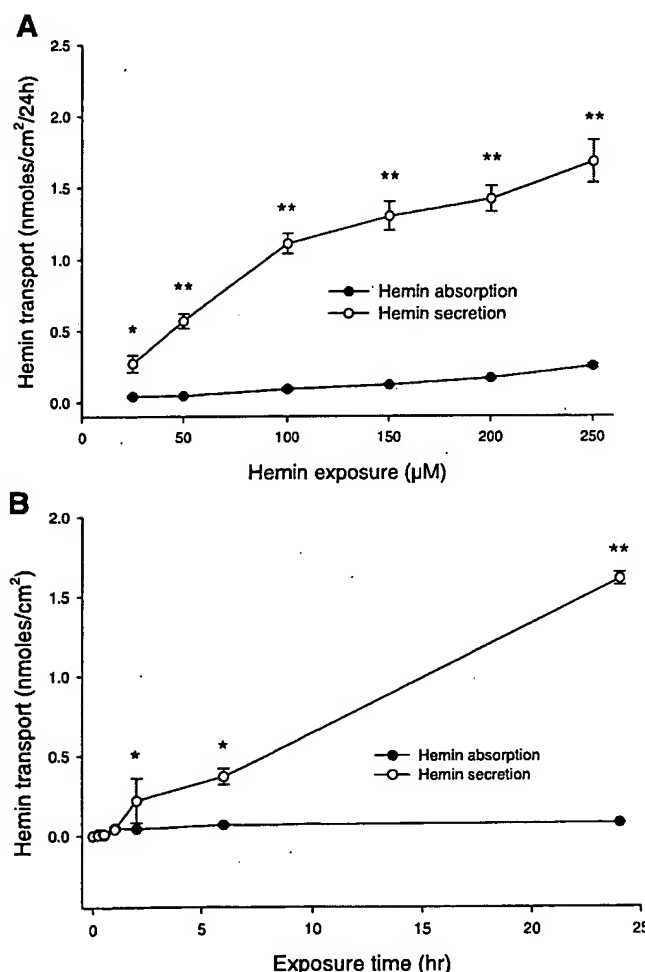


Fig. 3. Hemin transport in Caco-2 cells. Caco-2 cells grown on filters were treated with hemin from the apical side ( $\bullet$ ) or the basolateral side ( $\circ$ ), and hemin transport to the opposite side was determined spectrophotometrically. A: dose response: hemin moved in both directions, but transport from the basolateral to the apical side (secretion) was faster. B: time course: hemin secretion (100  $\mu$ M) and absorption (100  $\mu$ M) were examined at 15 min, 30 min, 1 h, 2 h, 6 h, and 24 h. Hemin absorption and secretion were very low and not different the first 1 h of exposure. Hemin secretion increased with time; absorption was stable after 2 h.  $n = 4$ ,  $*P < 0.05$ ;  $**P < 0.01$ .

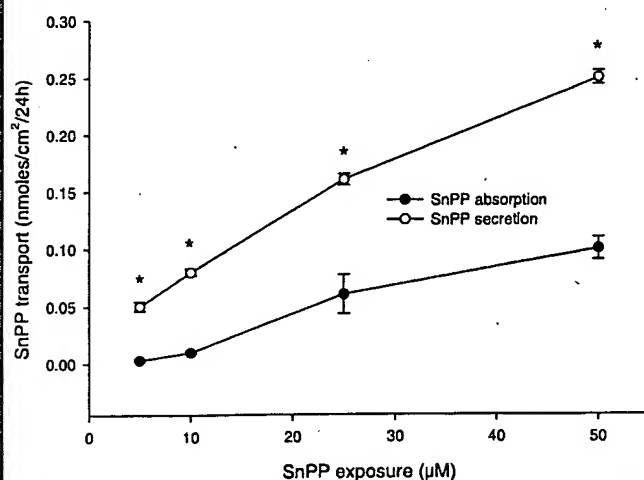


Fig. 4. Tin protoporphyrin (SnPP) transport in Caco-2 cells. Caco-2 cells were treated with SnPP from the apical side (●) or the basolateral side (○) for 24 h, and transport to the opposite side was determined spectrophotometrically. As with hemin, basolateral SnPP secretion was faster.  $n = 5$ ,  $*P < 0.01$ .

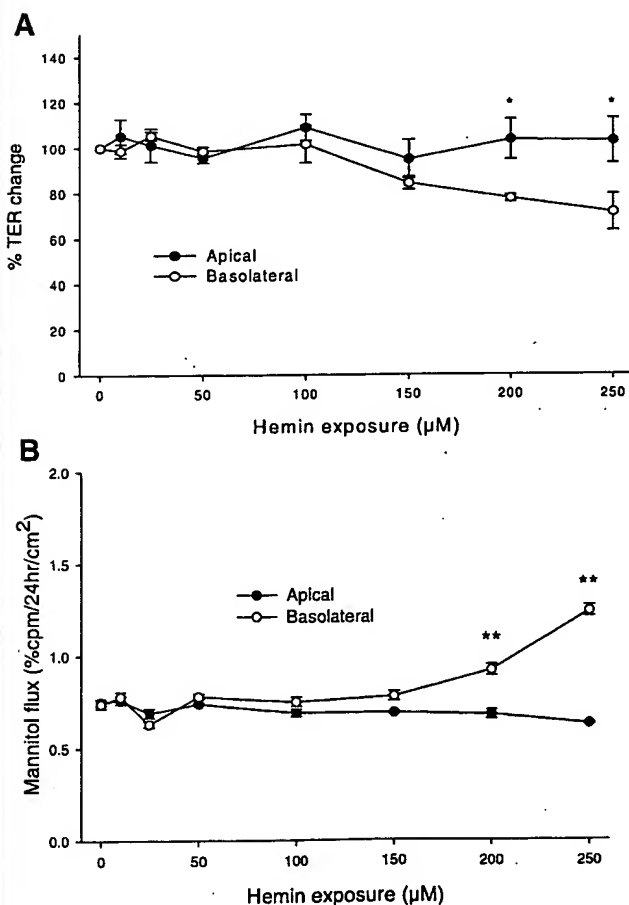


Fig. 5. Effect of hemin on Caco-2 cell paracellular permeability. Caco-2 cells were grown on filters and transepithelial resistance (TER) (A) or [ $^3$ H]mannitol (0.2 µCi/ml) flux (B) was measured after 24 h of treatment with hemin (10–250 µM) from the apical (●) or basolateral (○) side. At high concentrations ( $\geq 200$  µM), basolateral hemin reduced Caco-2 cell TER and induced [ $^3$ H]mannitol flux.  $n = 8$ ,  $*P < 0.05$ ;  $**P < 0.01$ .

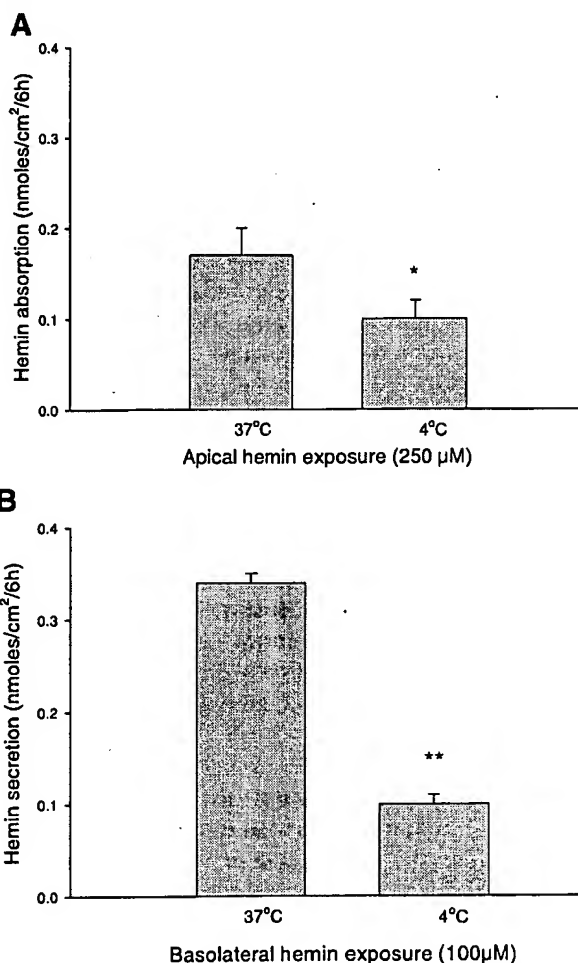


Fig. 6. Effect of temperature on hemin transport in Caco-2 cells. Confluent Caco-2 cells grown on filters were treated with hemin from the apical side (A) or the basolateral side (B) and hemin (nmol·cm<sup>-2</sup>·6 h<sup>-1</sup>) transport to the opposite side was determined spectrophotometrically. Low temperatures slowed hemin absorption and secretion.  $n = 11$  For apical hemin,  $n = 9$  for basolateral hemin;  $*P < 0.05$ ;  $**P < 0.01$ .

did not increase absorption of 10 µM SnPP ( $0.045 \pm 0.003$  vs.  $0.043 \pm 0.008$  nmol·cm<sup>-2</sup>·6 h<sup>-1</sup> after trypsin preincubation,  $n = 5$ ,  $P =$  not significant).

*Hemin-induced HO-1 expression shows polarity in other epithelial cell lines.* To determine whether the differential responses to hemin were present in other epithelial cell lines, we investigated hemin-induced HO-1 expression in two lung

Table 1. Hemin secretion occurs against a concentration gradient

Apical Chamber		Basolateral Chamber	
0 h	24 h	0 h	24 h
10	11.9±0.17	10	9.17±0.11*
25	27.2±0.1	25	22.8±0.6*
50	52.3±0.17	50	45.6±0.71*
100	113.2±0.82	100	97.5±0.55*

Values are means  $\pm$  SE in micromoles;  $n = 4$  cells. Confluent Caco-2 were treated with the same concentrations of hemin apically and basolaterally. After 24 h, hemin was more concentrated on the apical side, confirming that secretory transport occurred against a concentration gradient.  $*P < 0.01$ .



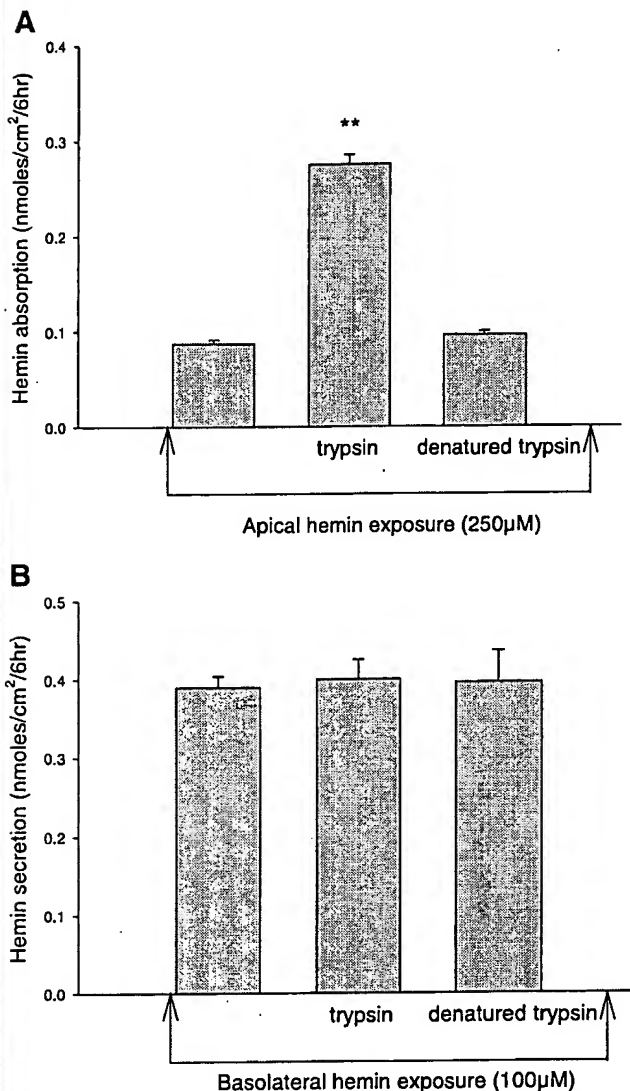


Fig. 7. Effect of trypsin on heme transport in Caco-2 cells. Confluent Caco-2 cells were grown on filters and incubated with trypsin (0.0025%)/EDTA (0.001%) for 5 min apically or basolaterally before hemin exposure on that side. Cells were then washed and exposed to hemin apically (A) or basolaterally (B) for 6 h. Trypsin increased apical hemin absorption by 3-fold, and had no effect on basolateral hemin secretion.  $n = 5$  For apical hemin,  $n = 11$  for basolateral hemin;  $**P < 0.01$

epithelial cell lines, Calu-3, H441, and one intestinal epithelial cell line, T84 cells. After 100 μM of hemin treatment for 24 h, HO-1 induction was more pronounced if hemin was applied from the basolateral side of cells (Fig. 8). This finding suggests that the polarity of hemin-induced HO-1 expression may be a general phenomenon for epithelial cells and not unique to Caco-2 cells.

#### DISCUSSION

With their ability to take up heme and induce HO-1, Caco-2 cells represent a good model to study the intestinal heme transport (6, 15, 44, 46). Because intestinal epithelial cells are primarily exposed to heme in vivo coming from the intestinal lumen, we hypothesized that the HO-1 induction would pri-

Table 2. Trypsin does not affect TER or [<sup>3</sup>H]mannitol flux

	Hemin (100 μM BL)		Hemin (250 μM A)	
	trypsin (-)	trypsin (+)	trypsin (-)	trypsin (+)
TER, (Ω·cm <sup>2</sup> )	1188 ± 44.7	1337 ± 23.6	1097 ± 5.6	1068 ± 6.8
[ <sup>3</sup> H]mannitol flux, %cpm·cm <sup>-2</sup> ·h <sup>-1</sup>			0.9 ± 0.09	0.75 ± 0.08

Values are means ± SE of 9 cells for basolateral (BL) hemin and 4 cells for apical (A) hemin. Confluent Caco-2 were treated with hemin (250 μM) apically or 100 μM basolaterally for 6 h after 5 min preincubation with trypsin (0.0025%)-EDTA (0.001%). Trypsin preincubation did not change transepithelial resistance (TER) and [<sup>3</sup>H]mannitol flux.  $P =$  not significant.

marily occur after apical hemin exposure. Contrary to our expectations, hemin-induced HO-1 expression was far greater if the treatment was applied to the basolateral side of the cells. This effect was also observed in lung epithelial cells. To our knowledge, differential HO-1 expression in response to hemin has not been reported in epithelial cells. In the previous studies that used Caco-2 cells as an in vitro model for heme transport, cells were grown on tissue culture plates, not on porous membranes (6, 46), and differential hemin exposure was not studied (15).

Similar to in vivo intestinal epithelium, Caco-2 cells respond to many substances differently if given from the apical rather than the basolateral side. For example, Caco-2 cells respond to the growth stimulating effects of epidermal growth factor, only if they are exposed from the basolateral side (5). Cholic acid, an irritant bile acid, causes toxicity if given basolaterally, but not apically (32). Caco-2 cells show selective apical transport for certain amino acids and bile acids, and they respond to some bacterial toxins only from the apical side (4, 27, 30).

The reason for the differential heme responses we observed in Caco-2 cells is not known, but it cannot be explained by a difference in total cellular heme contents after apical or basolateral exposure. It seems likely that heme goes to different subcellular compartments after apical or basolateral exposure, because its effects on HO-1 induction were quite different. We postulate that this differential HO-1 response is related to different heme transport mechanisms on the apical vs. basolateral side of the cells.

We observed that hemin passes intact to the opposite side of the cells and the transport is much faster if cells are exposed to hemin from the basolateral side. The intact absorption of heme

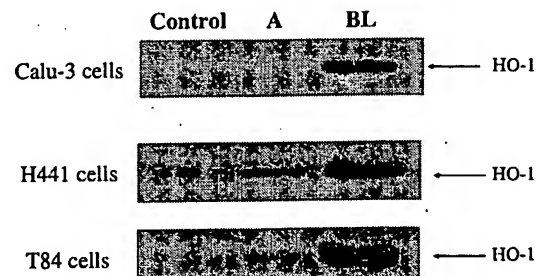


Fig. 8. Differential induction of HO-1 in alveolar and intestinal epithelial cells. Calu-3, H441, and T84 cells grown on filters were treated with hemin 100 μM from the apical (A) or the basolateral side (BL) for 24 h. Immunoblots were performed. Hemin-dependent induction of HO-1 in 3 cell lines was greater if hemin was applied basolaterally. The results are representative of 3 separate experiments.

from the intestines into the circulation has been demonstrated in guinea pigs (11) but was not seen in dogs or rats (35, 47). To our knowledge, the intact absorption or secretion of heme has not been examined in humans. Presumably, intestinal epithelial cells absorb heme as an intact metalloporphyrin and degrade it with HO-1, and the inorganic iron formed from heme degradation is handled similarly to nonheme iron acquired by the cell through the diet or body stores (9). Follett et al. (15) studied the absorption of  $^{59}\text{Fe}$ -hemin in Caco-2 cells by measuring the radioactivity on the basolateral side, but did not determine whether the detected  $^{59}\text{Fe}$  was associated with intact hemin.

Because hemin was 100% protein bound in our experimental conditions and precipitated in the absence of proteins in the medium, we postulated that protein binding played an important role in hemin transport. Protein binding of heme is physiologically relevant because heme is bound to plasma proteins in vivo (34) and to globins in the diet (8). The absorption of heme iron is enhanced in Caco-2 cells in the presence of globin (15), supporting the importance of protein binding in intestinal heme transport. Binding of heme to plasma proteins, mainly to hemopexin is known to reduce heme-mediated oxidative damage by making free heme less available (3, 21) and enhancing its endocytosis into cells expressing hemopexin receptors (42). Hemopexin-mediated heme uptake is well described in hepatocytes (43), as well as promyelocytic HL 60 cells (1) and human T-lymphocytes (41). The role of protein binding on intestinal heme uptake has not been described, and hemopexin receptors have not been reported in intestinal epithelial cells.

Heme is incorporated into important cellular enzymes (catalase, peroxidases, cytochrome *c*, NOS, NADPH oxidase, etc.) and all mammalian tissues contain an inducible HO-1 (39). Heme induces HO-1 expression in vivo and in vitro, which will then degrade the prooxidant heme, negate its toxic effects, and produce important by-products such as carbon monoxide, biliverdin, and bilirubin (29). It is not known whether induction of HO-1 has a role in hemin secretion or two processes run independently but in parallel. The role of HO-1 in intestinal heme transport is not investigated, but its role in iron reutilization is well known. HO-1 null mice develop hypoferrremia and iron overload in their liver and kidney (36). Microcytic hypochromic anemia, renal, and hepatic iron deposition were also observed in a child with HO-1 deficiency (48).

In our study, cell-associated hemin increased rapidly over the first 2 h of hemin exposure and reached a plateau after 6 h. These early time points should not be significantly impacted by cellular heme metabolism because HO-1 is not yet induced significantly (44). Although apical hemin associated with cells more efficiently than basolateral hemin at these earlier time points, hemin absorption and secretion were still low and not significantly different. The role of cellular heme metabolism and endogenous heme levels on intestinal heme uptake and transport cannot be estimated from these studies and will be investigated in the future.

Hemin secretion occurred largely via transcellular pathways ( $<200\ \mu\text{M}$ ) and it was an active process, because it occurred against a concentration gradient. Heme secretion by epithelial cells has not been previously reported. One earlier study suggests that the intestines can acquire heme from the basolateral side (45). Intravascular heme infusion increased the amount of heme and HO-1 in monkey intestine, suggesting the

presence of basolateral heme uptake in intestinal epithelial cells (45). We speculate that heme secretion and HO-1 induction may be mechanisms to dispose of circulating heme. Differential HO-1 expression in response to heme seems to occur in other epithelial cells too.

The mechanism for differential absorptive and secretory transport is not known, but it may be due to different expression of heme transporters on the apical and basolateral membranes. The trypsin experiments raise an intriguing possibility. Trypsin may have changed the conformation of an apical transporter or a regulatory protein, therefore allowing cells to absorb more heme. It is highly unlikely that trypsin increased hemin absorption by increasing paracellular permeability; we did not observe a decrease in TER or increase in mannitol flux with trypsin. Of note, trypsin is an endogenous protease, secreted from the pancreas into the intestinal lumen. It will be of interest to determine whether there is a role for endogenous proteases in regulating heme absorption.

We observed that another porphyrin, SnPP was also more rapidly secreted than absorbed. Unlike hemin, SnPP absorption did not increase after trypsin incubation. These results raise the possibility that intestinal epithelial cells possess a heme-specific transport system that does not recognize all porphyrins. These differential responses may be in place to protect humans from toxic effects of heme, by increasing its transport from the basolateral side and controlling its absorption apically.

In summary, heme is capable of being acquired and transported in both absorptive and secretory directions in polarized Caco-2 cells. Secretion is via an active metabolic/transport process. Trypsin increases the apical to basolateral transport, suggesting the presence of a protein that negatively regulates heme absorption by the apical membrane or a protein that is activated by cleavage with trypsin. These events may be important mechanisms to control heme absorption and prevent iron overload in humans.

#### ACKNOWLEDGMENTS

Present address of B. E. Britigan: Dept. of Internal Medicine, Veterans Affairs Medical Center-Cincinnati and University of Cincinnati College of Medicine.

#### GRANTS

This work was supported by National Institute of Health Grants DK-63135 (to A. Uc), DK-96001 (to J. B. Stokes), and AI-34954 (to B. E. Britigan), as well as grants from the Department of Veteran's Affairs (to J. B. Stokes and B. E. Britigan).

#### REFERENCES

1. Alam J and Smith A. Receptor-mediated transport of heme by hemopexin regulates gene expression in mammalian cells. *J Biol Chem* 264: 17637–17640, 1989.
2. Alvarez-Hernandez X, Nichols GM, and Glass J. Caco-2 cell line: a system for studying intestinal iron transport across epithelial cell monolayers. *Biochim Biophys Acta* 1070: 205–208, 1991.
3. Balla G, Vercellotti GM, Muller-Eberhard U, Eaton J, and Jacob HS. Exposure of endothelial cells to free heme potentiates damage mediated by granulocytes and toxic oxygen species. *Lab Invest* 64: 648–655, 1991.
4. Berger V, Larondelle Y, Trouet A, and Schneider YJ. Transport mechanisms of the large neutral amino acid L-phenylalanine in the human intestinal epithelial Caco-2 cell line. *J Nutr* 130: 2780–2788, 2000.
5. Bishop WP and Wen JT. Regulation of Caco-2 cell proliferation by basolateral membrane epidermal growth factor receptors. *Am J Physiol Gastrointest Liver Physiol* 267: G892–G900, 1994.

6. Cable JW, Cable EE, and Bonkovsky HL. Induction of heme oxygenase in intestinal epithelial cells: studies in Caco-2 cell cultures. *Mol Cell Biochem* 129: 93–98, 1993.
7. Carpenter CE and Mahoney AW. Contributions of heme and nonheme iron to human nutrition. *Crit Rev Food Sci Nutr* 31: 333–367, 1992.
8. Conrad ME, Cortell S, Williams HL, and Foy AL. Polymerization and intraluminal factors in the absorption of hemoglobin-iron. *J Lab Clin Med* 68: 659–668, 1966.
9. Conrad ME and Umbreit JN. Iron absorption and transport—an update. *Am J Hematol* 64: 287–298, 2000.
10. Conrad ME, Umbreit JN, and Moore EG. Iron absorption and transport. *Am J Med Sci* 318: 213–229, 1999.
11. Conrad ME, Weintraub LR, Sears DA, and Crosby WH. Absorption of hemoglobin iron. *Am J Physiol* 211: 1123–1130, 1966.
12. Daskaleros PA, Stoeber JA, and Payne SM. Iron uptake in *Plesiomonas shigelloides*: cloning of the genes for the heme-iron uptake system. *Infect Immun* 59: 2706–2711, 1991.
13. Ebert PS, Frykholm BC, Hess RA, and Tschudy DP. Uptake of hematin and growth of malignant murine erythroleukemia cells depleted of endogenous heme by succinylacetone. *Cancer Res* 41: 937–941, 1981.
14. Ebert PS, Frykholm BC, Hess RA, and Tschudy DP. Characteristics of hematin uptake in malignant, embryonic and normal cells. *Cancer Biochem Biophys* 6: 157–165, 1983.
15. Follett JR, Suzuki YA, and Lonnerdal B. High specific activity heme-Fe and its application for studying heme-Fe metabolism in Caco-2 cell monolayers. *Am J Physiol Gastrointest Liver Physiol* 283: G1125–G1131, 2002.
16. Galbraith RA. Heme binding to Hep G2 human hepatoma cells. *J Hepatol* 10: 305–310, 1990.
17. Galbraith RA, Sassa S, and Kappas A. Heme binding to murine erythroleukemia cells. Evidence for a heme receptor. *J Biol Chem* 260: 12198–12202, 1985.
18. Grasbeck R, Kouvonen I, Lundberg M, and Tenhunen R. An intestinal receptor for heme. *Scand J Haematol* 23: 5–9, 1979.
19. Grasbeck R, Majuri R, Kouvonen I, and Tenhunen R. Spectral and other studies on the intestinal haem receptor of the pig. *Biochim Biophys Acta* 700: 137–142, 1982.
20. Grasset E, Pinto M, Dussaux E, Zweibaum A, and Desjeux JF. Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. *Am J Physiol Cell Physiol* 247: C260–C267, 1984.
21. Gutteridge JM and Smith A. Antioxidant protection by hemopexin of haem-stimulated lipid peroxidation. *Biochem J* 256: 861–865, 1988.
22. Hallberg L, Bjorn-Rasmussen E, Howard L, and Rossander L. Dietary heme iron absorption. A discussion of possible mechanisms for the absorption-promoting effect of meat and for the regulation of iron absorption. *Scand J Gastroenterol* 14: 769–779, 1979.
23. Hallberg L, Brune M, Erlandsson M, Sandberg AS, and Rossander-Hulten L. Calcium: effect of different amounts on nonheme- and heme-iron absorption in humans. *Am J Clin Nutr* 53: 112–119, 1991.
24. Hallberg L, Hulten L, and Gramatkovski E. Iron absorption from the whole diet in men: how effective is the regulation of iron absorption? *Am J Clin Nutr* 66: 347–356, 1997.
25. Hallberg L and Rossander L. Effect of soy protein on nonheme iron absorption in man. *Am J Clin Nutr* 36: 514–520, 1982.
26. Hansen MB, Nielsen SE, and Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 119: 203–210, 1989.
27. Hidalgo IJ and Borchardt RT. Transport of bile acids in a human intestinal epithelial cell line, Caco-2. *Biochim Biophys Acta* 1035: 97–103, 1990.
28. Hidalgo IJ, Raub TJ, and Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 96: 736–749, 1989.
29. Keyse SM and Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci USA* 86: 99–103, 1989.
30. Kovbasnjuk OE. Role of lipid rafts in Shiga toxin 1 interaction with the apical surface of Caco-2 cells. *J Cell Sci* 114: 4025–4031, 2001.
31. Light WR III, and Olson JS. Transmembrane movement of heme. *J Biol Chem* 265: 15623–15631, 1990.
32. Lowes S and Simmons NL. Human intestinal cell monolayers are preferentially sensitive to disruption of barrier function from basolateral exposure to cholic acid: correlation with membrane transport and trans-epithelial secretion. *Pflügers Arch* 443: 265–273, 2001.
33. Majuri R and Grasbeck R. A rosette receptor assay with haem-microbeads. Demonstration of a haem receptor on K562 cells. *Eur J Haematol* 38: 21–25, 1987.
34. Morgan WT, Liem HH, Sutor RP, and Muller-Eberhard U. Transfer of heme from heme-albumin to hemopexin. *Biochim Biophys Acta* 444: 435–445, 1976.
35. Parmley RT, Barton JC, Conrad ME, Austin RL, and Holland RM. Ultrastructural cytochemistry and radioautography of hemoglobin-iron absorption. *Exp Mol Pathol* 34: 131–144, 1981.
36. Poss KD and Toneyawa S. Heme oxygenase 1 is required for mammalian iron reutilization. *Proc Natl Acad Sci USA* 94: 10919–10924, 1997.
37. Raffin SB, Woo CH, Roost KT, Price DC, and Schmid R. Intestinal absorption of hemoglobin iron-heme cleavage by mucosal heme oxygenase. *J Clin Invest* 54: 1344–1352, 1974.
38. Rao RK, Li L, Baker RD, Baker SS, and Gupta A. Glutathione oxidation and PTPase inhibition by hydrogen peroxide in Caco-2 cell monolayer. *Am J Physiol Gastrointest Liver Physiol* 279: G332–G340, 2000.
39. Ryter SW and Tyrrell RM. The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free Radic Biol Med* 28: 289–309, 2000.
40. Sayegh R, Auerbach SD, Li X, Loftus RW, Husted RF, Stokes JB, and Thomas CP. Glucocorticoid induction of epithelial sodium channel expression in lung and renal epithelia occurs via trans-activation of a hormone response element in the 5'-flanking region of the human epithelial sodium channel alpha subunit gene. *J Biol Chem* 274: 12431–12437, 1999.
41. Smith A, Eskew JD, Borza CM, Pendrak M, and Hunt RC. Role of heme-hemopexin in human T-lymphocyte proliferation. *Exp Cell Res* 232: 246–254, 1997.
42. Smith A and Hunt RC. Hemopexin joins transferrin as representative members of a distinct class of receptor-mediated endocytic transport systems. *Eur J Cell Biol* 53: 234–245, 1990.
43. Smith A and Morgan WT. Hemopexin-mediated transport of heme into isolated rat hepatocytes. *J Biol Chem* 256: 10902–10909, 1981.
44. Uc A and Britigan BE. Does heme oxygenase-1 have a role in Caco-2 cell cycle progression? *Exp Biol Med (Maywood)* 228: 590–595, 2003.
45. Vreman HJ, Rodgers PA, and Stevenson DK. Zinc protoporphyrin administration for suppression of increased bilirubin production by iatrogenic hemolysis in rhesus neonates. *J Pediatr* 117: 292–297, 1990.
46. Worthington MT, Cohn SM, Miller SK, Luo RQ, and Berg CL. Characterization of a human plasma membrane heme transporter in intestinal and hepatocyte cell lines. *Am J Physiol Gastrointest Liver Physiol* 280: G1172–G1177, 2001.
47. Wyllie JC and Kaufman N. An electron microscopic study of heme uptake by rat duodenum. *Lab Invest* 47: 471–476, 1982.
48. Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, and Kolzumi S. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest* 103: 129–135, 1999.